ADAPTATION TO CERTAIN TENSIONS OF OXYGEN AS SHOWN BY GONOCOCCUS AND OTHER PARASITIC AND SAPROPHYTIC BACTERIA*

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Practically all our knowledge concerning the respiration of bacteria has been thoroughly reviewed by Kruse,[1] and an especially illuminating discussion of this subject has been given by Meyer.[2] We shall not go into detail concerning the influence of oxygen tension on sporulation, germination, division, and motility. We would remind the reader, however, that chiefly through the investigation of Chudiakow, Winogradski, Porodko, Wundt, and others, it became known that the optimal conditions for the growth of any single species do not depend so much on the presence or absence of oxygen as on its tension, and further, that through the work of Beijerinck[3] we became acquainted with a group of bacteria which thrive only at a partial atmospheric tension of oxygen—the microaerophiles. Among these may be mentioned the bacteria studied in bean infusions by Beijerinck; the sulfur bacteria, the peculiar behavior of which was reported by Winogradski; Amylobacter butylicum, which, according to Beijerinck's observation, only grows and ferments wort under anaerobic conditions but grows better at a lower temperature in peptone-starch water under more nearly aerobic conditions; B. abortus, which von Bang and Stribolt found required a partial tension for its growth; a bacillus isolated from fowl diphtheria by Müller;[4] and certain pyogenic streptococci, as shown by Wittneben.[5]

It seems remarkable to us that greater attention has not been paid to the oxygen requirements of parasitic bacteria. A few recent experiences have suggested to us that the cultivation of many of the unknown viruses of infectious diseases may depend more on the presence of the right oxygen tension than on the composition of the

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[1] Allgemeine Mikrobiologie, 1910.
[2] Centralbl. f. Bakteriol., I, O., 1909, 49, p. 305.
[3] Ibid., 1893, 14, p. 827.
[4] Ibid., I, O., 1906, 41, pp. 521, 621.
[5] Ibid., 1907, 44, p. 96.

artificial medium. Rosenow,[6] however, has repeatedly noted that cultures made from rheumatic joints, appendicitis, etc., are more apt to yield positive results when tall columns of broth or agar are used. Of cultures from rheumatic joints he says: "That the oxygen requirement is the chief factor to explain this difference in my results and the negative results of others is indicated also by the fact that the colonies never developed above 0.5 cm. from the top and never below 2 cm. from the bottom of the agar tubes. The largest number of colonies developed between 1.5 cm. from the top and 3.5 cm. from the bottom." This oxygen-tension requirement was lost in the Rosenow strains on further cultivation (presumably aerobic or anaerobic subcultures) and he further notes[7] that "after cultivation from one to eight months, the capacity to grow at a low temperature, the sensitiveness to oxygen, the excessive production of acid in dextrose broth, and the simultaneous affinity for joints, endocardium, and myocardium are found to have largely or entirely disappeared." Again, in connection with bacteriologic examination of the glands draining the lesions in arthritis deformans Rosenow states that "all the streptococcal forms isolated have shown a marked preference for anaerobic conditions of growth in the primary culture."[8]

We record here the details of our discovery[9] that the gonococcus is a partial-tension organism. We also describe a partial-tension clostridium; and a partial-tension bacterium, from a human knee joint, resembling B. abortus. None of these three organisms will grow anaerobically, but they throw off aerobic variants from their partial-tension growths. We further show that Leptothrix innominata of the human mouth has a very wide range of oxygen tension and we record some observations which tend to show that B. typhosus becomes adapted to partial-tension growth within the body.

TECHNIC

We have produced partial oxygen pressure in our cultures almost entirely by the method first suggested by Nowak,[10] but instead of sealing the cultures in a jar along with one or more slant cultures of B. subtilis, we have attached a recently inoculated slant culture of this organism to the tube in which partial tension is required, by means of rubber tubing. Modifications of Nowak's method have been suggested by Horton[11] and Giltner.[12] It will also be recalled

[6] Jour. Infect. Dis., 1914, 14, p. 62.
[7] Ibid., p. 66.
[8] Jour. Am. Med. Assn., 1914, 62, p. 1146.
[9] Wherry and Oliver: Lancet-Clinic, 1916, 115, p. 306.
[10] Ann. de l'Inst. Pasteur, 1908, 22, p. 541.
[11] Jour. Infect. Dis., 1914, 15, p. 22.
[12] Centralbl. f. Bakteriol., R., 1914, 63, p. 522.

that Theobald Smith and Fabyan[13] showed that other bacteria—B. megatherium and B. coli for example—could be used in place of B. subtilis. Our method is an easy one and enables one to re-attach freshly inoculated tubes of B. subtilis at various intervals. Micro-organisms which grow slowly may not appear at all unless B. subtilis tubes are changed daily so as to maintain the required tension. In this connection it is well to remember that B. subtilis may still multiply at an atmospheric pressure of 10 mm., tho not at 5 mm. (Chudiakow).

In making partial-tension plates we have inverted the inoculated plate upon a glass plate on which is fastened (with a small piece of plasticine) a smaller open Petri dish containing a freshly inoculated culture of B. subtilis.. The inverted dish should be high enough to avoid any contact with the smaller dish. The edges of the inverted dish are then sealed with plasticine as recommended by Lentz[14] for the growth of anaerobes. Plates made in this way and sealed to pieces of window glass of suitable size, may be stacked in the incubator.

THE GONOCOCCUS A NATURAL MICROAEROPHILE

The recognition of the fact that the gonococcus is a partial-tension organism renders its cultivation an easy matter. In a study of vulvovaginitis we had the usual experience with a variety of media but were finally successful in obtaining aerobic strains of the gonococcus from 4 cases of vulvovaginitis in children and from the urethral pus of an adult male. Having been particularly impressed with the work of W. Blair M. Martin,[15] we used the medium and criteria established by him for the cultivation and recognition of the gonococcus. We were disappointed, however, in invariably obtaining only a few colonies of the gonococcus when the inoculated pus showed that myriads of organisms were present. The following experiences which we have had more recently, explain the discrepancy.

(a) Boy, 4 years old. Acute purulent urethritis with extreme phimosis, which had begun one week before. Smears showed numerous intracellular gram-negative diplococci resembling gonococci. One loopful of pus was smeared by the successive-stroke method over 3 slants of +0.5 Martin's medium containing sterile pleuritic fluid. Two sets of 3 tubes each were inoculated in this way. One tube which was inoculated with a loopful of pus, was kept at partial tension. Incubation 37 C.

In 24 hours the partial-tension tube showed hundreds of minute translucent gonococcus-like colonies, which when subcultured corresponded culturally to the gonococcus. None of the aerobic tubes showed any growth in 24 hours and no gonococcus-like colonies

[13] Centralbl. f. Bakteriol., I, O., 1912, 61, p. 549.
[14] Ibid., 1910, 53, p. 358.
[15] Jour. Path. and Bacteriol., 1911, 15, p. 76.

appeared at all, tho in from 48 to 72 hours there were a few colonies of the urethral diphtheroid.

Two days later this experiment was repeated with in each case one loopful of the urethral pus for the inoculation of each slant. No gonococcus-like colonies appeared on the aerobic slants, but on slants kept at partial tension they were very numerous in 48 hours.

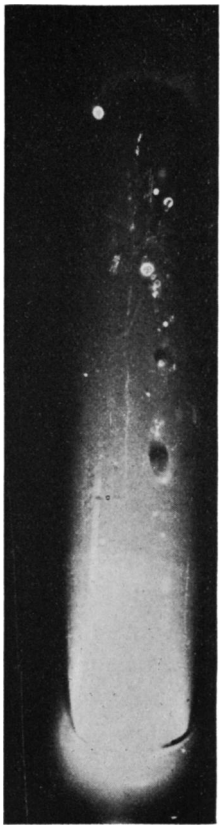

Figure 1

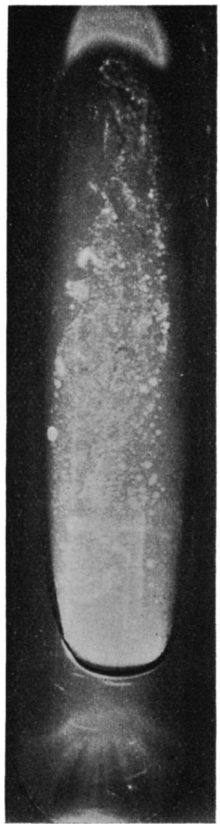

Figure 2

Figure 3

Figs. 1 and 2. Aerobic and partial-tension cultures from the male urethra in gonorrhea. One loopful of pus spread on each slant. Incubation on + 0.5 Martin's pleuritic medium for 24 hours at 37 C. The vast majority of the colonies on the partial-tension slant (2) are gonococcus colonies. × ½.

Fig. 3. Isolated gonococcus colonies on + 0.5 Martin's pleuritic medium, partial tension, at 37 C. for 4 days. Note delicate translucent edge. Natural size.

An aerobic strain of the gonococcus was derived from one of these partial-tension subcultures as follows:

Aerobic subcultures were made along with partial-tension subcultures 24 and 48 hours after isolation; the aerobic subcultures remained sterile during 8 days' observation. When the partial-tension strain was in its 8th subculture (8 days old), another attempt yielded an aerobic strain, which, however, grew rather poorly for the first two or three aerobic transfers and then grew as luxuriantly as the partial-tension strain. This aerobic strain was found to be dead after 6 days' incubation at 37 C.

We have not made many viability tests on the partial-tension strains, but one culture kept at partial tension for 15 days at 37 C., yielded almost as luxuriant a growth on subculture as one gets on transplanting a young culture; that is, an almost confluent layer. Another partial-tension strain isolated from a case of vulvovaginitis, after 19 consecutive days' incubation at partial tension at 37 C. yielded, on transplant, a luxuriant growth.

We have not succeeded in obtaining an anaerobic strain from the partial-tension cultures. Anaerobic subcultures kept for 4 days at 37 C. and then placed at partial tension do not yield growth.

The 7-year-old sister of this little boy, with whom he slept, had no vaginal discharge and aerobic and partial-tension cultures yielded no gonococcus-like colonies. The parents denied having, or having had, gonorrhea. No cultures were made in their case.

(b) Girl, 11 years old. Suspected rape. Profuse purulent vaginal discharge showing a large number of extracellular and fewer intracellular gram-negative diplococci resembling gonococci. Cultures were made on 4 slants of +0.5 Martin's pleuritic medium. Three of these tubes were kept at partial tension and the fourth aerobic. After 24 hours at 37 C. all the partial-tension tubes showed hundreds of minute translucent colonies which when subsequently studied seemed to correspond with the gonococcus. The aerobic tube, after 24 hours, gave 8 translucent colonies of gram-negative cocci which resembled the gonococcus. When this aerobic culture was 24 hours old, aerobic and partial-tension subcultures were made from a single isolated colony. The aerobic subculture showed luxuriant growth, while that at partial tension showed only a few scattered colonies. Microscopic examination of these subcultures revealed apparently pure cultures of gram-negative diplococci.

(c) Man, 26 years old. Subacute gonorrhea of some months' standing. Moderate urethral discharge obtained by expression. Direct smears showed numerous cocci resembling gonococci. Three slants of +0.5 Martin's pleuritic medium were inoculated, each with a loopful of pus. Two were kept at partial tension and the third aerobic.

The two former showed hundreds of typical translucent colonies composed of gram-negative cocci; the aerobic culture yielded 5 opaque colonies and 4 translucent colonies, which were not examined.

A PARTIAL-TENSION CLOSTRIDIUM

We encountered this organism first in glucose-broth blood cultures from a case of epilepsy. It forms spores which resist flowing steam for 15 minutes on 3 successive days or autoclaving at 15 pounds' pressure for some time under 30 minutes, but is killed at this temperature in 30 minutes. Dr. M. B. Cohen in this laboratory isolated it from glucose broth which was supposed to have been autoclaved at 15 pounds for 30 minutes, but in a special series of experiments in which he operated the autoclave himself, it was found not to resist this temperature. It has never been encountered in broth cultures without the glucose and a special series of experiments made by Dr. Cohen proved that the spores are present in glucose. Spores of both aerobic and partial-tension strains exist. The aerobic strains grow freely on incubation of the broth. But other tubes or flasks which appear sterile on aerobic incubation may contain the partial-tension spores and these are induced to grow with difficulty. If sterile blood is added to such a flask of glucose broth and then it is inoculated with Staphylococcus aureus, the spores apparently germinate slowly and after a week or so, the rods may be found in rather moderate numbers in the sediment but not in the supernatant fluid. Subcultures from this sediment usually yield the staphylococcus only, but occasionally after several days' incubation a growth of the clostridium will appear in the staphylococcus growth. When such a growth (on human-blood agar) containing spores is separated from the staphylococci by heating to 80 C. and planting in a deep tube of +0.5 Martin's medium containing pleuritic fluid, the clostridium develops slowly as a hazy line just beneath the surface of the medium. Subcultures from this line of growth on slants of the same medium yield no growth aerobically but luxuriant growth at partial tension. However, subcultures into another deep tube yield a similar growth just beneath the surface. The partial-tension slant culture remains true to type through several subcultures on Martin's pleuritic medium.

An aerobic strain was derived from it as follows: It was noted that transplanting from the hazy layer just beneath the surface of a deep shake culture would yield no aerobic growth; but if such a tube, the surface of which had been broken by the needle, was incubated for a

day or two longer, growth appeared on its surface and this could then be transplanted aerobically.

The partial-tension strain kept on Martin's pleuritic medium, differs in several points from the naturally or artificially derived aerobic strains. It forms longer and thicker rods, which often give the granulose reaction with Gram's iodin solution, and spore-formation does not take place until days have elapsed; altho spore-formation appears within 24 hours at 37 C. in partial-tension subcultures on +1 human-blood agar, as it does also in any culture of the aerobic strain.

A PARTIAL-TENSION BACTERIUM FROM A HUMAN KNEE JOINT

The patient was pregnant, had a purulent discharge from the vagina containing gram-negative diplococci resembling gonococci, and developed an arthritis of the left knee joint. This was aspirated and the sediment from the rather clear fluid was planted on +0.5 Martin's pleuritic medium and incubated aerobically. No growth. About 10 days later one of us superintended the collection of a second sample. This was markedly purulent. No bacteria could be found microscopically tho a number of staining methods were employed. The centrifugated sediment was planted on +0.5 Martin's pleuritic-agar slants and +1 human-blood-agar slants and incubated under aerobic and anaerobic conditions. These all remained sterile. Two plate cultures were made from the sediment in +0.5 Martin's pleuritic medium; one was incubated aerobically and the other at partial tension. The aerobic plate showed no growth, while thousands of very minute colonies apeared throughout the partial-tension plate. The organism proved to be a minute rod, which morphologically and culturally is much like B. abortus. We expect to make a further comparative study of this organism.

PARTIAL-TENSION CULTURES OF LEPTOTHRIX INNOMINATA

The organism was isolated by making anaerobic plates with material scraped from the human gum line. The anaerobic culture yields partial-tension subcultures but it will not grow aerobically. In deep-tube shake cultures it grows well throughout the medium and almost to its surface but not on the surface. As there is some doubt about this organism's having been cultivated before, we shall report its characters in detail separately.[16]

[16] Jour. Infect. Dis., 1916, 19, p. 299.

PARTIAL-TENSION CULTURES OF BACILLUS TYPHOSUS

The following single experiment suggests that the vast majority of typhoid bacilli growing in the body tissues are well over on the partial-tension side, and indicates for the first time, as far as we are aware, the extreme grade which the septicemia may attain in this disease.

Girl, ill with continued fever for 7 days. Rose spots on abdomen. Eight cubic centimeters of blood drawn from vein. Four large bottles of + 1 broth inoculated with 1 c.c. of blood each. The rest of the blood was discharged into sterile 1.5% sodium-citrate solution. This last was centrifugated at high speed for about 10 minutes and the sediment hemolyzed with sterile distilled water and centrifugated again for one-half hour. Temperature about 25 C. (We realize that in making any accurate numerical count of the number of bacilli, the blood should have been chilled thoroughly between these operations. But in spite of the fact that the blood, after withdrawal, was at about 25 C. for nearly 1 hour before it was plated, we hardly think that this amount of incubation will account for the enormous numbers found. Further, we fail here to account for the very large number of bacilli which were present in the citrate plasma and which grew when this was placed in broth and deep tubes of agar.) The sediment was planted in several deep tubes of Martin's medium, in + 1 broth, and a quantity which represented about 1.5 c.c. of blood was plated in each of 2 six-inch plates containing + 1 Martin's medium and pleuritic fluid. One of these plates was incubated aerobically and the other kept at partial tension.

The aerobic plate showed thousands of colonies, but in the partial-tension plate they were present probably by the millions—in fact, they were so crowded that it was hopeless to attempt to count them. This partial-tension plate became contaminated after 48 hours by B. subtilis, which grew up one side of the plate and over its surface.

A partial-tension strain of B. typhosus was isolated as follows:

Some of the washed sediment of the patient's blood had been incubated aerobically in + 1 broth for 48 hours at 37 C. There was a slight scum on the surface composed of apparently a pure culture of actively motile gram-negative bacilli. A little of the growth near the bottom of this tube was removed with a sterile capillary pipet and diluted with sterile salt solution. This dilution was then smeared with a loop on slants of + 0.3 and + 1 Martin's pleuritic medium. One-half of these were incubated aerobically and the rest kept at partial tension.

In 24 hours the aerobic slants showed numerous isolated colonies of a fairly uniform size (1-1.5 mm.). The partial-tention slants showed equally numerous 1-1.5 mm. colonies, but between these were almost as many pinpoint-sized colonies. Subcultures from these very minute colonies showed that they were of the partial-tension strain. The subcultures were made from the +0.3 Martin's pleuritic slants to

tubes of the same medium. For example: (a) In aerobic and partial-tension subcultures from the same 1-mm. colony on the partial-tension slant the growth was rather much alike in the two tubes with perhaps more growth on the aerobic. (b) Some of the minute pinpoint colonies on the partial-tension culture were mixed together and aerobic, partial-tension, and anaerobic subcultures made. The aerobic subculture yielded a delicate layer of growth, while the partial-tension subculture was covered by a luxuriant confluent layer.

The anaerobic subculture showed discrete, well isolated colonies—just as if all the bacilli in the minute partial-tension colonies were not fitted for anaerobic growth.

Litmus-lactose-agar plates made from the original blood-sediment-broth culture, from which these strains were isolated, yielded apparently a pure culture of typhoid-like colonies. It is perhaps a matter of chance here, but the vast majority of these plate colonies were deep colonies. A culture from one of these colonies fermented dextrose, levulose, galactose, maltose, and mannite with acid-production but no gas, and did not attack lactose or saccharose. This test was made on +1 Martin's pleuritic medium. The sugars, etc., were sterilized for 20 minutes at 15 pounds' pressure and added to the medium aseptically just before solidifying, as recommended by Martin.

The partial-tension strain was a very actively motile bacillus resembling the aerobic strain. When grown at partial tension on the same batch of sugar media used for testing the aerobic strain, it did not attack the afore-mentioned carbohydrates and alcohols during 4 days' observation. In these partial-tension cultures the formation of many filamentous forms during 24 hours' growth was a noteworthy observation, as we believe that this explains the formation of filaments in fluid cultures. We have also noted the tendency to formation of filaments in 24-hour-old colonies of B. coli on +0.5 Martin's pleuritic medium. The gross appearance of such colonies is not unlike that presented by the colonies of Leptothrix innominata.

DISCUSSION

It would seem probable that the majority of bacteria actively multiplying within the tissues of a host are adapted to a pressure of less than 21% oxygen. When transplanted from the tissues to artificial media under aerobic conditions, only a few bacteria, which are favored either by situation or by associated micro-organisms, become adapted to aerobic growth. However, as we have seen, when such bacteria are

brought out under conditions of reduced oxygen tension, the majority thrive well. What effect this observation may have on the cultivation of some of the hitherto unrecognized infectious agents remains to be seen. Such partial-tension parasites, when cultivated on such a medium as the sodium-phosphate pleuritic agar of Martin, become adapted to, or tenaciously cling to, the type of respiration required on this medium. They are with difficulty led over to the aerobic type of respiration by transplants on the same medium. If they grow at all under what appears to be aerobic conditions, close examination of the growth will show that they are not growing on the surface of the slant but within the surface layer. Further aerobic subcultures on the same medium have failed in our hands as a rule. In fact we have found that it is advisable to choose a medium of entirely different composition when one wishes to get an aerobic strain from a partial-tension one; for example, one may transplant aerobically to beef-extract agar or to human-blood agar or to maltose agar successfully, tho in the first place these media were unsuitable for the primary partial-tension growth from the tissues of the host.

The fact that the partial-tension strains of the bacillus from the knee joint and that of B. typhosus failed to attack glucose under partial tension, but attacked it vigorously when grown aerobically on the same medium, is certainly worth considering a little further. In the past, the tendency among medical bacteriologists has been to grow aerobically mass cultures of micro-organisms isolated from disease processes in the body, and to imply that the metabolic activities of such bacteria are comparable to those in the body. Theoretically it seems to us to be probable that the majority of micro-organisms thriving within the body exist under partial-oxygen tension and that some become very definitely adapted to such lowered oxygen concentration. Is it not possible that the partial-tension mode of nutrition in vitro is more nearly that which the micro-organism follows in the body of the host? If so, does failure to take this into consideration account for our inability in the past to recognize toxin-production by many species in vitro? Does this mode of nutrition lead to the building up of a bacterial cell body which has a very different chemical composition from the ordinary aerobic and anaerobic strains we have worked with in the past? The work of Bordet[17] on the antigenic properties of old and recently isolated strains of the whooping cough bacillus; the greater immunizing value of a "body strain" of B. pestis as shown by Row-

[17] Centralbl. f. Bakteriol., I, O., 1912, 66, p. 276.

land,[18] and the numerous recent observations by Rosenow on the tendency of certain strains of bacteria to localize at certain sites in the body, all point to the great importance of a closer study of the nutritional requirements of parasitic bacteria. Certainly past experience points clearly to the supreme influence of oxygen tension and temperature upon variation. Beijirinck[19] has shown that aerobic, partial-tension, and anaerobic strains of lactic-acid ferments may be derived by successive transfer from the top, middle portion, or bottom of fermented milk to corresponding regions of oxygen tension in the inoculated material. He points out very clearly that the character of the fermentation, that is, the end products, is controlled chiefly by temperature and oxygen pressure. It is unnecessary to quote in detail his numerous observations on variation but we are tempted to quote the following: "Variations in oxygen pressure above or below that most favorable to vital functions is undoubtedly one of the chief factors and these ferments only continue to display constant specific characters when continuously cultivated at a certain oxygen pressure—otherwise these characters disappear and in fact, or apparently, new ones originate. In wholly different groups of bacteria corresponding facts may be observed, hence their fundamental significance." Again, "That temperature is a decisive factor in the production of variants is shown by experience, for, prolonged cultivation above the optimum temperature gives rise to forms differing from the original stock."

[18] Jour. Hyg., 1914, 13, Suppl. 3, p. 403.
[19] Proc. Roy. Acad., 1907, 10, p. 17.